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Identification of an HIV-1 Clade A Envelope That Exhibits Broad Antigenicity and Neutralization Sensitivity and Elicits Antibodies Targeting Three Distinct Epitopes

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Broadly neutralizing antibodies (bNAbs) PG9 and PG16 were isolated from an International AIDS Vaccine Initiative (IAVI) Protocol G subject infected with human immunodeficiency virus type 1 (HIV-1) clade A. Both antibodies are highly potent and neutralize greater than 70% of viruses tested. We sought to begin immunogen design based on viral sequences from this patient; however, pseudoviruses prepared with 19 envelope sequences from this subject were resistant to neutralization by PG9 and PG16. Therefore, we used a bioinformatics approach to identify closely related viruses that were potentially sensitive to PG9 and PG16. A most-recent common ancestor (MRCA) sequence for the viral envelope (Env) was determined and aligned with 99 subtype A gp160 sequences from the Los Alamos HIV database. Virus BG505.W6M.ENV.C2 (BG505) was found to have the highest degree of homology (73%) to the MRCA sequence. Pseudoviruses prepared with this Env were sensitive to neutralization with a broad panel of bNAbs, including PG9 and PG16. When expressed by 293T cells as soluble gp120, the BG505 monomer bound well to both PG9 and PG16. We further showed that a point mutation (L111A) enabled more efficient production of a stable gp120 monomer that preserves the major neutralization epitopes. Finally, we showed that an adjuvanted formulation of this gp120 protein elicited neutralizing antibodies in rabbits (following a gp120 DNA vaccine prime) and that the antisera competed with bNAbs from 3 classes of nonoverlapping epitopes. Thus, the BG505 Env protein warrants further investigation as an HIV vaccine candidate, as a stand-alone protein, or as a component of a vaccine vector.

The development of a vaccine to prevent AIDS is the best hope for controlling the epidemic that has led to more than 30 million people worldwide being infected with human immunodeficiency virus type 1 (HIV-1). A vaccine approach that reduces viral load would certainly be beneficial, but one that elicits sterilizing immunity would be preferred. For many years, only a few anti-HIV-1 broadly neutralizing antibodies (bNAbs) were known, including 2G12 (anticarbohydrate antibody) (1–3), 2F5 (anti-gp41 membrane-proximal external region [MPER] antibody) (1, 3) and 4E10 (anti-gp41 MPER antibody) (4) prepared from human hybridomas, and b12 (anti-CD4bs antibody) (5, 6) and D5 (anti-gp41 N-heptad repeat [NHR] antibody) (7), which were isolated from phage display libraries. Several studies have shown that these first-generation bNAbs can protect animals from viral infection (8–18), providing evidence that a vaccine eliciting a significant population of such antibodies will protect individuals from infection.

The International AIDS Vaccine Initiative (IAVI) initiated a global program called Protocol G that sought to identify potent and broadly neutralizing sera from HIV-1-infected patients. Analysis of >1,800 different serum samples identified ~1% as "elite" neutralizers that could neutralize pseudoviruses representing 4 different clades with high potency (19). B cells were isolated from elite patients to screen for individual cells secreting potent neutralizing Abs (20). By screening the culture supernatants from about 30,000 activated memory B cells from one clade A-infected African elite neutralizer, 2 highly potent, broadly neutralizing monoclonal antibodies (PG9 and PG16) were identified (20). PG16 is relatively trimer specific, whereas PG9 binds trimer preferentially but can bind monomeric gp120 from at least a dozen viral isolates. By expanding this work to include 4 additional donors, 18 additional broadly neutralizing antibodies (PGT121 to PGT123, PGT125 to PGT131, PGT135 to PGT137, and PGT141 to PGT145) were discovered (21). Of those antibodies, only the PGT141 to PGT145 family exhibits characteristics similar to PG9 and PG16. In work at the NIH Vaccine Research Center (VRC), a panel of broadly neutralizing sera was screened for binding to a "resurfaced" gp120 antigen, which was designed to enhance selection of antibodies specific for the CD4bs by replacing non-CD4bs, surface-exposed residues with those from simian immunodeficiency virus (SIV) (22). From this work, the potent and broadly neutralizing anti-CD4bs antibodies VRC01, VRC02, and VRC03 (22) and PGV04 (21) were discovered. More recently, a broad and potent anti-MPER antibody was described (23) that lacks the autoreactivity associated with 2F5 and 4E10. Recent structural stud-

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ies have now identified, at high resolution, the molecular determinants of the neutralization-sensitive epitopes (22, 24–27), giving hope that immunogens that present these conserved sites of vulnerability could form the basis for an effective vaccine.

Our goal has been to identify a native Env sequence that presents the maximum number of conserved neutralization-sensitive epitopes, or sites of vulnerability, to serve as the starting point for vaccine development. We have identified a clade A Env (BG505) that binds to bNAbs representative of most of the known gp120 neutralizing antibody classes when tested by enzyme-linked immunosorbent assay (ELISA). Of note, gp120 monomers from BG505 bind to conformation-sensitive antibodies (PG9 and PG16), suggesting that it retains certain structural features of the native Env trimer. Here, we determine the antigenicity profile of soluble gp120 monomers and cell surface-expressed BG505 Env trimers and demonstrate that pseudoviruses prepared using this Env are sensitive to neutralization with a broad panel of bNAbs, including PG9 and PG16. We further show that a point mutation introduced at position L111 of BG505 Env enables more efficient production of soluble gp120 monomers that contain determinants bound by at least 3 different classes of neutralizing antibody. Finally, we show that an adjuvanted formulation of this gp120 protein elicits neutralizing antibodies in rabbits following a gp120 DNA vaccine prime and that sera from immunized rabbits compete with the bNAbs for binding to 3 distinct epitopes on gp120.

MATERIALS AND METHODS

Phylogenetic analysis. Ninety-nine subtype A gp160 sequences were retrieved from the Los Alamos HIV database (http://hiv.lanl.gov) and aligned to the 19 unique virus Env sequences isolated from the PG9/PG16 donor (subject 024) using HMMER 3.0 (http://hmmer.org). Subtype assignment and lack of intrasubtype recombination was confirmed using the SCUEAL algorithm (28). A maximum likelihood phylogeny was reconstructed with GARLi version 2.0 (http://garli.nescent.org). Next, branch lengths were fitted under the HIV-1-specific protein substitution model (29), and the most-recent common ancestor (MRCA) sequence of viruses isolated from subject 024 were inferred using marginal maximum likelihood in HyPhy (30).

Selection of viral Env clones. Libraries were created from plasma sample collected from subject 024 at the time of PG9 isolation (064V1) and approximately 8 months later (064V2) by PCR amplification of full-length envelope genes from cryopreserved plasma. The quasispecies present in the PCR products were cloned into a plasmid expression vector, eETV. To select individual Env clones, we retransformed the plasmid preparation containing the gene populations, plated the plasmid library on agar plates, and picked 48 clones from each individual. The plasmid DNA was isolated from each clone and used to create a stock of pseudovirus particles that were then screened for infectivity and neutralization by PG9 and PG16. Plasmids containing cloned envelope glycoproteins were sequenced using fluorescently labeled dideoxynucleotides using capillary electrophoresis sequencing devices (Applied Biosystems, Foster City, CA). Eight to 12 sequencing primers were employed to generate bidirectional coverage of each sequence.

Envelope protein production. Soluble gp120 proteins were produced by transient expression from transfected HEK 293T/17 (293T) cells (ATTC CRL-11268) or from the *N*-acetylglucosaminetransferase I (GnTI)-deficient HEK293S cell line (ATCC CRL-3022). Genes encoding clade A gp120 (BG505 or BG505 L111A) were codon optimized and synthesized at GeneArt, Life Technologies, and inserted into pCI-neo under the control of the human cytomegalovirus (CMV) promoter. The Env signal sequence was replaced with the corresponding region from human CD5 to improve expression, and a hexa-his tag was added to the C terminus for use in protein purification. Plasmids expressing clade C gp120 (16936 or 16096-V55) were designed similarly except that the plasmid vector was pcDNA3.1 (Invitrogen). Env gp120 secreted from transfected cells was purified by metal ion chromatography (IMAC) using a HisTrap column (GE). Alternatively, protein preparations were purified by affinity chromatography with a mannose-specific *Galanthus nivalis* lectin (GNL) agarose column (31) (Sigma). The lectin column was equilibrated with phosphate-buffered saline (PBS), pH 7.4. Elution was performed with 0.5 M methyl-alpha-D-mannopyranoside (Sigma). The elution buffer was exchanged for PBS by diafiltration, and the protein was stored at −80°C. SDS-polyacrylamide gel electrophoresis, size exclusion chromatography, and Western blotting using a panel of Abs recognizing individual domains of the purified Envs were used for more detailed assessment of protein quality.

DNA vaccines. Plasmids encoding gp120 used for DNA vaccination are described above. Aldevron LLC (Fargo, ND) performed large-scale production and purification of vaccine material.

Animals. Female New Zealand White rabbits were purchased and maintained at Covance (Denver, PA) under specific pathogen-free conditions. Rabbits were anesthetized, and the area over the *vastus lateralis* muscle of the anterior hind limbs was shaved before immunization with 250 μg HIV Env DNA by electroporation. Briefly, a TriGrid electrode array (Ichor Medical Systems, Inc., San Diego, CA) was percutaneously placed into the target muscle, and DNA was delivered into the muscle via an autoinjection device. Electroporation was applied to the injection site 10 s after initiation of the intramuscular (i.m.) injection (75 to 500 V/cm, maximum duration of 45 ms, maximum of 6 pulses). Alternatively, animals were immunized with 50 μg of HIV Env protein formulated with 75 Isco units of ISCOMATRIX adjuvant (CSL). Animals were primed with DNA twice at weeks 0 and 4, before protein boost immunizations at weeks 14 and 20, or vice versa. The Covance Institutional Animal Care and Use Committee approved all experimental protocols.

Pseudovirus production and antibody neutralization assays. HIV-1 envelope gp160 expression plasmids used for producing pseudoviruses (except for clade A BG505) were obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH, or were designed based on sequences in the database and produced by GeneArt, an Invitrogen company. Plasmid used for preparing clade A BG505 pseudoviruses was pCI-neo-BG505 gp160-deltaCT, which contains a hexa-his tag in place of the gp160 cytoplasmic domain. Pseudovirions were produced by cotransfecting 293T/17 cell monolayers with plasmid encoding Env and the pSG3deltaEnv (backbone) vector and titrated on TZM-bl cells in quadruplicate as previously described (32). Antisera from immunized rabbits were tested for their ability to neutralize HIV-1 using a panel of HIV-1 pseudoviruses: SF162, JRCSF, 93IN905, 94UG103, 92TH021, BG505, 92BR020, and SVAMuLV (negative control). These pseudoviruses were chosen because they are representatives of clades A, B, C, and CRF01_AE; vary in their neutralization sensitivities; and have been used previously to successfully identify other bNAbs (19). Neutralization assays were conducted by infecting the TZM-bl cell line with ~150,000 relative light units (RLU) of each pseudovirus, which were preincubated with serial dilutions of heat-inactivated sera from immunized rabbits before addition of cells. Following 48 h of incubation, cells were lysed and luciferase activity was determined using a microtiter plate luminometer and BriteLite Plus reagent (PerkinElmer). Neutralizing activity of sera can be identified as a significant reduction in the luciferase signal relative to control wells (33, 34).

Rabbit sera were also tested in the A3R5 assay, in which neutralization was measured as a reduction in *Renilla* luciferase (LucR) reporter gene expression after infection with HIV-1 Env.IMC.LucR viruses in A3R5 cells. A3R5 cells (35) were obtained from Jerome Kim and Robert McLinden at the U.S. Medical HIV Research Program (MHRP). This is a human $CD4^+$ lymphoblastoid cell line (CEM/A3.01) (36) that was engineered at the MHRP to express CCR5. Infectious molecular clones of HIV-1 carrying the entire ectodomain of the virus of choice and a Tat-regulated LucR

reporter gene (37) were obtained from Christina Ochsenbauer and John Kappes at the University of Alabama, Birmingham. Assay stocks of Env. IMC.LucR viruses were prepared by transfection in 293T cells (3) and were titrated in A3R5 cells. The A3R5 assay was performed as described previously (38). Briefly, a dose of virus that generates approximately 50,000 RLU after 4 days of infection was incubated with serial 3-fold dilutions of test sample in duplicate for 1 h at 37°C. Exponentially dividing A3R5 cells (90,000 cells in medium containing 25 μg/ml DEAE dextran) were added to each well. One set of control wells received cells and virus (virus control), and another set received cells only (background control). After 4 days of incubation, luciferase activity was measured using the ViviRen live cell substrate as described by the supplier (Promega) and a Victor3 luminometer (PerkinElmer). Neutralization titers are the dilution at which RLU were reduced by 50% compared to virus control wells after subtraction of background RLUs. Pre- and postimmune sera from each animal were assayed side by side in the A3R5 assay. Taking the variability inherent in this cell-based assay into account, titers in the postimmune sample that are ≥3 times the titer in the preimmune sample are considered positive for HIV-specific neutralizing activity.

Analysis of Env protein bound to aluminum phosphate. Env proteins were formulated for immunogenicity studies by adsorption to aluminum phosphate (alum) adjuvant (Adju-Phos; Brenntag Biosector, Frederikssund, Denmark), and the antigenicity of these formulations was determined by flow cytometry (39). Env proteins were incubated with 100 μg of alum for 30 min at 37°C and then blocked with PBS containing 3% bovine serum albumin (BSA) prior to staining with various antibodies at 1 μg per ml. The alum particles were washed with PBS-1% BSA and then stained for 25 min at room temperature (RT) in the dark with Alexa 555-conjugated goat anti-human IgG (Invitrogen catalog number A21433, used at 1:500) in a 1% BSA-PBS solution. After being washed, 20 to 30,000 particles were acquired in the fluorescent channel phycoerythrin (PE) using an LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software package (TreeStar, Cupertino, CA).

Analysis of Envs expressed by transient transfection. 293T/17 (ATCC) cells (8×10^6 per T75 flask) were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 25 mM HEPES buffer, and gentamicin. The monolayers were transfected with 30 μg of pCI-neo encoding BG505 L111A gp160-deltaCT, which contains a hexa-his tag in place of the natural gp160 cytoplasmic domain. Transfections were performed with TransIT (Mirus Bio) or Fugene 6 (Promega) transfection reagent according to the manufacturer's instructions. The medium was changed the next day, after which incubation was continued for 2 more days at 37°C. The cells were harvested using dissociation medium (Invitrogen) to prepare single-cell suspensions. Cells were stained with Live/Dead aqua fluorescent reactive dye (1:1,000; Invitrogen) for 20 min and then incubated with various human monoclonal antibodies (MAbs) for 30 min at room temperature. The cells were washed with fluorescent-activated cell sorting (FACS) buffer (PBS and 5% FBS) and then incubated with a 1:500 dilution of secondary antibody (Alexa Fluor-conjugated goat anti-human IgG; Invitrogen) for 30 min before being analyzed by flow cytometry as described above.

ELISA. ELISA was performed as described elsewhere (40) using plates coated with JRCSF gp120 antigen at a concentration of 2 μg/ml. Serial titration of test sera was performed with a starting dilution of 1:100, and individual preimmune sera were used as negative controls. Following a 1-h incubation period and three washes, a 1:500 dilution of alkaline phosphatase-conjugated AffiniPure goat anti-rabbit IgG (Jackson Immuno Research) was added. After a 1-h incubation, plates were washed three times with PBS with 0.1% Tween 20 and twice with distilled water. Alkaline phosphatase substrate (Sigma-Aldrich) was added, and plates were read 30 min later at a wavelength of 405 nm. An analysis of recombinant Env proteins was done manually using the same protocol except that antigen capture was done using an anti-Penta His monoclonal antibody (Qiagen, Valencia, CA).

Multiplexed competition antibody binding assay. To determine epitope specificity, we developed a competition-binding assay (CBA) based on Meso Scale Discovery (MSD) Sector Imager technology. MSD uses electrochemiluminescence (ECL) to detect binding events in a unique and sensitive manner with a >4-log dynamic range. The goal of the CBA is to determine whether sera from vaccinated animals contain Abs recognizing epitopes similar to those bound by well-characterized Env-specific Abs. The Abs PG9, PGT126, PGV04, b6, 17b, and 2F5 were each spotted onto MSD multiarrays. Dilutions of sera were preincubated for 1 h at 37°C with an excess of soluble biotinylated BG505 L111A gp120 or with a clade C gp120 chimera based on isolate 16096 with a V1 to V3 loop substitution from subtype 16055, a clade C isolate that we term 16936-V55. This mixture was then transferred to an MSD plate blocked with PBS containing 3% BSA and incubated for 1 h at room temperature. Plates were washed with PBS containing 0.02% Tween 20 before incubation with a streptavidin-Sulfo tag reporter reagent. Plates were read using the Sector Imager 2400. A reduction in signal reflects the presence of Abs in serum that competed with spotted Abs for binding to gp120. An advantage of this assay configuration is that it detects binding to soluble antigen, which may possess a more native structure compared with plate-bound antigen.

RESULTS

Identification of HIV Env sequences that express the PG9 epitope. The bNAbs PG9 and PG16 were isolated from subject 024 (41) in IAVI's Protocol G. Nineteen separate clade A Env sequences were detected in samples from this subject, representing 13 distinct haplotypes. Subject 024 Env sequences were derived from plasma samples collected at two time points, one contemporaneous with the isolation of PG9 and the other later. Pseudoviruses made with the corresponding Envs were resistant to neutralization with PG9 and PG16, suggesting that mutants had evolved in the subject 024 virus population that eliminated the relevant antibody-binding determinants. Therefore, we used computational methods to find theoretical progenitor virus sequences that would be closely related and encode Env that was potentially sensitive to neutralization with PG9 and PG16. The phylogeny developed from analyzing the 13 unique Env sequences from subject 024 is shown in Fig. 1. The most-recent common ancestor (MRCA) sequence is quite divergent from any database isolates and most closely related to the BG505.W6M.ENV.C2 sequence (42) (GenBank accession no. ABA61516/DQ208458), which had a minimum amino acid evolutionary distance estimated at 0.43 substitutions/site. The BG505 Env sequence has a 73% amino acid homology to the reconstructed MRCA sequence of the Protocol G subject—the highest among all GenBank sequences.

Four clade A Envs (97CM.MP570, 92RW026, 92RW0008, BG505.W6M.ENV.C2) with the highest similarity to MRCA were characterized further. Supernatant from transfected 293T/17 cells was used as a source of gp120 proteins, which were tested subsequently for binding to PG9/PG16 by ELISA. Only the BG505 gp120 bound PG9 and PG16 (data not shown). An ELISA conducted with BG505 gp120 and multiple Env-specific monoclonal antibodies is shown in Fig. 2. Subsequently, pseudovirus was prepared using the BG505 Env to determine its sensitivity to neutralization with a panel of human antibodies, including PG9 and PG16. As shown in Table 1, the BG505 pseudovirus was neutralized well by most of the bNAbs in our panel, including PG9, PG16, and PGT145, which also recognizes a quaternary neutralizing epitope (QNE) on HIV-1 (21). The BG505 pseudovirus also was neutralized by antibodies targeting other known sites of vulnera-

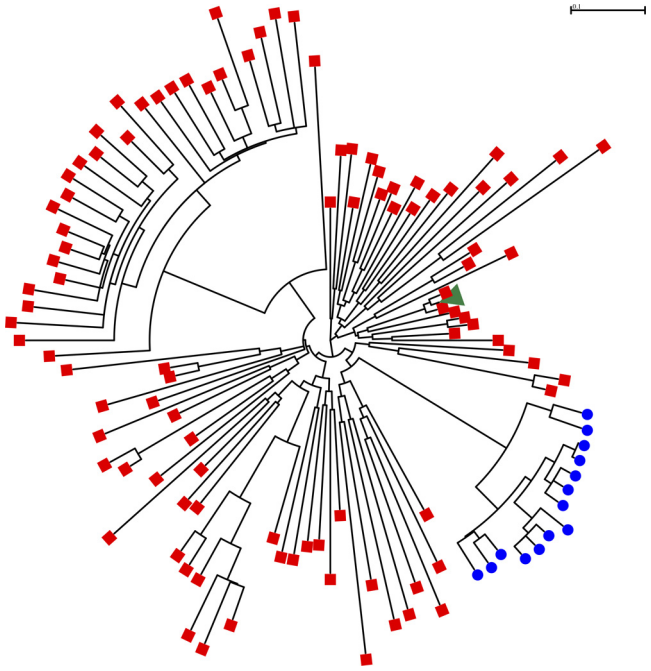

FIG 1 Maximum likelihood phylogeny with branch lengths estimated using the HIV-Between amino acid substitution model with site-to-site rate variation modeled by a 3-bin general discrete distribution. The phylogenetic tree shows isolates from subject 024, the PG9/16 donor (41) (blue circles), background subtype A sequences (red squares), and the BG505 sequence (green triangle). The scale at the top is 0.1 substitutions per amino acid residue.

TABLE 1 Neutralization titers (IC_{50}) against the BG505 (clade A) pseudovirus in the TZM-bl assay

Antibody	IC_{50} (μg/ml)
bNAbs	
b12	>25
2F5	0.46
4E10	0.89
2G12	>25
PG9	0.11
PG16	<0.01
PGV04	0.05
VRC01	0.06
PGT 121	0.06
PGT 122	0.09
PGT 123	5.37
PGT 125	<0.01
PGT 126	0.26
PGT 130	0.06
PGT 135	18.16
PGT 136	> 25
PGT 145	< 0.01
Control Abs	
D20	>25
15E	>25
23B	>25
A32-7	>25
F10	>25
17B	>25
T8	>25
b6	>25
8C7	>25
5B11	>25
EH21	>25
F105	>25

bility on Env, including the CD4bs (VRC01 and PGV04), the V3 glycan (PGT125 to PGT128), and glycan-only epitopes (PGT121 to PGT122), but not by two of the first-generation antibodies, b12 and 2G12 (Table 1).

During BG505 gp120 purification from transfected HEK 293 cell culture supernatants, a significant fraction of the protein aggregated, making it difficult to produce soluble monomer. It has previously been reported that a single amino acid substitution (L111A) can prevent dimer formation (43), so this mutation was introduced into BG505. The resulting protein, BG505 L111A, was shown to form stable gp120 monomers as assessed by SDS-PAGE performed under reducing and nonreducing conditions and Western blotting (Fig. 3). Pseudovirus prepared with the BG505 L111A mutant remained infectious and was sensitive to neutralization by the same antibodies that neutralized the wild-type BG505 pseudovirus (data not shown).

Antigenicity of BG505 variants. Initial antigenicity was assessed with an antigen-capture ELISA using gp120 from transfected cell culture supernatants. ELISAs were conducted with a panel of Env-specific human antibodies as described elsewhere (40). As shown in Fig. 2, the BG505 gp120 Env produced from 239T/17 cells bound PG9 and PG16 antibodies in addition to several antibodies directed to the CD4bs and to glycan-dependent epitopes recognized by the PGT series of Abs. BG505 antigenicity also was influenced by the cell line used for production. When gp120 was expressed in 293S GnTI$^-$ cells, which lack *N*-acetylglucosaminyltransferase I activity (44), binding of

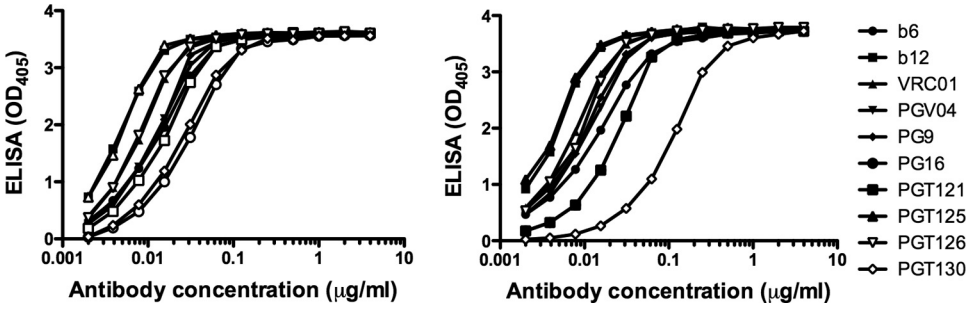

FIG 2 ELISA showing binding of the indicated human antibodies to BG505 L111A gp120 expressed in HEK 293T cells (A) or in HEK 293S GnTI$^-$ cells (B).

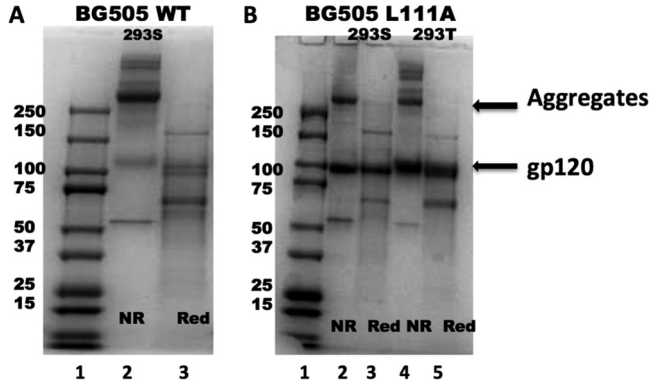

FIG 3 SDS-PAGE analysis of BG505 wild-type (wt) and BG505 L111A gp120 proteins under reducing and nonreducing conditions showing the mitigation of aggregate formation with the L111A mutation. (A) Wild-type BG505 gp120 purified by IMAC from HEK 293S cells. Lanes: 1, molecular weight standards; 2, nonreduced sample; 3, reduced sample. (B) BG505 L111A gp120 purified by IMAC. Lanes: 1, molecular weight standards; 2 and 3, protein purified from 293S cells; 4 and 5, protein purified from 293T cells; 2 and 4, nonreduced samples; 3 and 5, reduced samples.

PGT122 to PGT123 was reduced, as expected for antibodies that recognize a glycan epitope (Table 2). Unexpectedly, it was observed that PGT126 bound strongly to BG505 despite a threonine at position 332 instead of the glycosylated N residue that is part of the antibody-binding determinant for this family of antibodies (24). When BG505 T332 was mutated to N332, binding to PGT135 and PGT136 was achieved, indicating that glycosylation at this position is required for recognition by these antibodies (Table 2).

In order to use the same assay method to compare the antigenicity of gp120 monomers with gp160 trimers transiently expressed on the cell surface of 293T cells, we took advantage of the observation that BG505 gp120 monomers retain antigenicity after

TABLE 2 Summary of antibody binding specificity to BG505 mutants expressed in 293T versus 293S GnTI$^-$ cells

		ELISA response (OD_{405})			
		BG505 from 293S GnTI$^-$ cells		BG505 from 293T cells	
Antibody class	Antibody (2 μg/ml)	L111A	L111A + T332N	L111A	L111A + T332N
QNE	PG9	3.74	3.58	3.73	3.60
	PG16	3.72	3.55	3.70	3.37
CD4bs	b12	3.69	3.56	3.69	3.59
	b6	3.72	3.57	3.75	3.55
	VRC01	3.76	3.61	3.74	3.61
	PGV04	3.74	3.59	3.75	3.62
Glycan dependent	PGT121	1.67	3.63	3.74	3.55
	PGT122	0.06	3.61	3.72	3.54
	PGT123	0.23	3.59	3.61	3.58
	PGT125	3.76	3.64	3.74	3.62
	PGT126	3.76	3.59	3.75	3.56
	PGT130	3.61	3.62	3.56	3.53
	PGT135	0.00	3.57	0.00	**3.57**
	PGT136	0.00	3.61	0.00	**3.62**

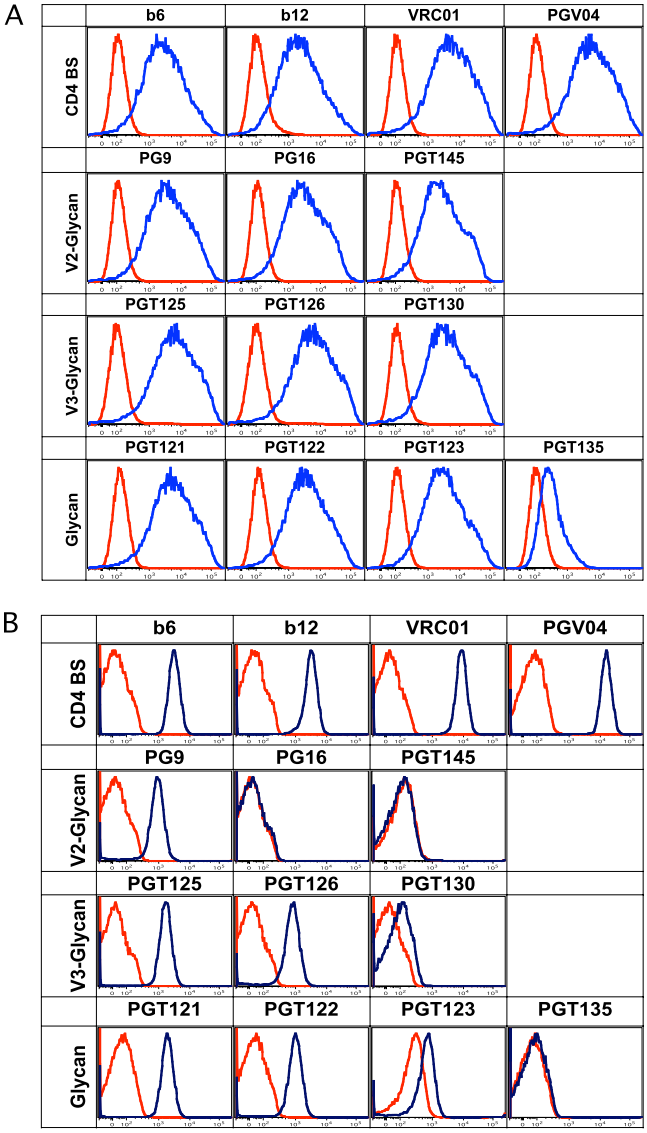

FIG 4 Antigenicity profile of BG505 L111A Env. BG505 L111A was expressed on the surface of 293T cells transfected with a gp160-deltaCT construct (A) or as gp120 protein adsorbed to aluminum phosphate particles (B) and stained with the indicated antibodies and analyzed by flow cytometry.

binding to aluminum phosphate (alum) particles, enabling their analysis by flow cytometry. After binding gp120 to alum, remaining binding sites were blocked with BSA, after which the particles were stained with various anti-Env primary Abs and a fluorochrome-labeled anti-human IgG second antibody. Analysis by flow cytometry indicated that fluorescence intensity was proportional to the quantity of gp120 protein bound to the alum particles. The antigenicity of BG505 L111A gp120 bound to alum particles was compared with that of BG505 L111A Env expressed on the surface of 293T cells. As shown in Fig. 4, all antibodies that bound to Env expressed on the cell surface (Fig. 4A) also reacted with gp120 protein (Fig. 4B), with the exception of PGT145, which recognizes a quaternary conformational epitope on Env trimers expressed on 293T cells but not displayed on gp120 monomers. PGT135 showed weak staining of BG505 L111A expressed

TABLE 3 Antibody response to HIV gp120 immunogens delivered in a prime-boost regimen against a heterologous gp120 protein[a]

Group ($n = 4$)	Immunogen		ELISA titer			
	Prime	Boost	PD-1 (wk 2)	PD-2 (wk 6)	PD-3 (wk 14)	PD-4 (wk 22)
1.1	BG505 L111A DNA	BG505 L111A protein	424	11,698	30,675	45,164
1.2	BG505 L111A DNA	BG505 L111A protein	6,643	29,084	43,275	58,237
1.3	BG505 L111A DNA	BG505 L111A protein	59	25,119	61,209	129,307
1.4	BG505 L111A DNA	BG505 L111A protein	272	11,285	65,202	40,773
GMT			**461**	**17,622**	**47,976**	**61,023**
2.1	16936 DNA	16936 protein	50	2,099	17,739	24,960
2.2	16936 DNA	16936 protein	97	356	20,296	28,995
2.3	16936 DNA	16936 protein	50	4,417	32,062	34,666
2.4	16936 DNA	16936 protein	50	1,957	52,460	46,499
GMT			**59**	**1,594**	**27,896**	**32,865**
3.1	BG505 L111A protein	BG505 L111A DNA	814	5,271	11,876	23,899
3.2	BG505 L111A protein	BG505 L111A DNA	448	3,261	11,306	16,700
3.3	BG505 L111A protein	BG505 L111A DNA	50	2,060	7,166	4,778
3.4	BG505 L111A protein	BG505 L111A DNA	50	3,197	58,076	32,316
GMT			**174**	**3,262**	**15,375**	**15,756**

[a] Immunogens were administered by intramuscular (i.m) injection at weeks 0 and 4 for priming and at weeks 12 and 20 for boosting. Sera collected at the indicated postdose (PD) time point were tested by ELISA for binding to JRCSF gp120. The dose for DNA vaccines was 250 μg i.m. delivered by electroporation, and the dose for protein vaccines was 40 μg i.m. formulated with ISCOMATRIX adjuvant. Negative titers were assigned a value of 50 for calculation of geometric mean titers (GMT).

on 293T cells but no binding to monomer. Adsorption of the gp120 protein to alum resulted in the loss of binding to PG16 (which was reactive in ELISA) but not to PG9 or other antibodies tested in Fig. 2.

BG505 L111A immunogenicity. BG505 L111A gp120 was assessed in a DNA prime-protein boost regimen, as this has been reported previously to improve antibody induction in comparison to gp120 vaccination alone (45). Rabbits were primed 2 times with gp120 DNA followed by two boosts with gp120 protein formulated with ISCOMATRIX adjuvant. For comparison, we included the clade C 16936 Env (46) gp120, which does not bind to PG9 or PG16. We also included a third (reverse prime-boost) group in which rabbits were primed with adjuvanted BG505 L111A gp120 protein and subsequently boosted with the corresponding gp120 DNA. The basis for testing the reverse prime-boost approach is that it was previously shown that this regimen resulted in higher and more durable antibody responses to the hepatitis B surface antigen in a mouse model (47). All DNA vaccinations were administered by electroporation using the Ichor TriGrid delivery system. Sera from vaccinated rabbits were tested by ELISA for binding to JRCSF, a heterologous clade B gp120 protein, and the results (Table 3) indicated that anti-gp120 antibody titers in group 1 (BG505 L111A DNA prime-protein boost) were ~2-fold higher than those attained with Env 16936 or in the reverse prime-boost group. Sera from the 3 groups were tested for neutralization of the tier 1 pseudoviruses SF162 (clade B) and 93IN905 (clade C) in the standard TZM-bl assay. As shown in Fig. 5, sera from the BG505 L111A DNA prime-protein boost group neutralized SF162 with 50% infective dose (ID_{50}) values significantly higher than those for sera from rabbits immunized with Env 16936 DNA prime-protein boost ($P = 0.029$, Mann-Whitney test). Neutralizing antibody titers to 93IN05, a moderately sensitive clade C virus, were similar for both DNA prime-protein

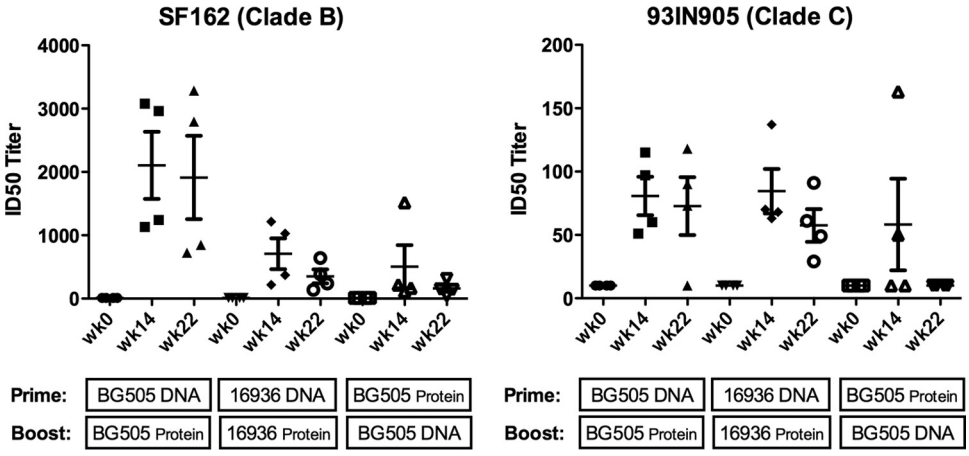

FIG 5 BG505 L111A vaccine elicits strong neutralizing antibodies to the sensitive pseudovirus SF162 (A) and to the moderately sensitive pseudovirus 93IN905 (B) in the standard TZM-bl assay. Rabbits were primed with DNA twice at weeks 0 and 4, before protein boost immunizations at weeks 14 and 20, or vice versa. Serum was assessed by TZM-bl neutralization assay at weeks 0, 14, and 22. Means and standard errors of the means (SEM) are shown; $n = 4$ subjects per time point.

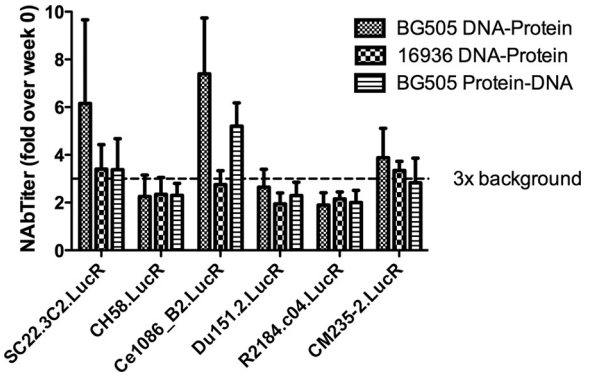

FIG 6 Neutralization titers determined with the A3R5 assay. Serum from groups of 4 rabbits immunized with the indicated vaccine regimen was assayed against 6 tier 2 reporter viruses (SC22.3C2.LucR.T2A.ecto, CH58.LucR.T2A.ecto, Ce1086_B2.LucR.T2A.ecto, Du151.2.LucR.T2A.ecto, R2184.c04.LucR.T2A.ecto, and CM235-2.LucR.T2A.ecto). Results are expressed as the mean fold rise of postvaccination titers relative to prevaccination titers for each virus and regimen. Pre- and postimmune sera from each animal were assayed side by side in the A3R5 assay. Taking the variability inherent in this cell-based assay into account, titers in the postimmune sample that are ≥3 times the titer in the preimmune sample are considered positive for HIV-specific neutralizing activity.

boost groups. Titers attained in the reverse prime-boost group were low against each pseudovirus tested in the TZM-bl assay.

Subsequently, we used the more sensitive A3R5 assay (48) to look for neutralization of six tier 2 viruses representing clade B (SC22.3C2.LucR.T2A.ecto, CH58.LucR.T2A.ecto), clade C (Ce1086_B2.LucR.T2A.ecto, Du151.2.LucR.T2A.ecto), and CRF01-AE (R2184.c04.LucR.T2A.ecto, CM235-2.LucR.T2A.ecto). Because some preimmune sera had high background activity against some viruses, we determined the ratio of postvaccination over prevaccination titers for each sample. Based on historical data, neutralization titers of >3-fold over background are considered significant. As shown in Fig. 6, sera from the BG505 DNA prime-protein boost group exhibited elevated neutralization titers for 2 of 6 viruses (SC22.3C2 and Ce1086), whereas sera from the 16936 prime-boost group had little or no activity against any of the 6 viruses in the panel. Sera from the BG505 L111A reverse prime-boost group had demonstrable neutralizing titers against 1 of 6 viruses (Ce1086).

As traditional ELISA and virus neutralization assays do not address the specificity of antibodies elicited in vaccinated animals, we developed a novel competition binding assay (CBA) to determine the relative levels of vaccine-induced antibodies to different regions of vulnerability on Env (49). These regions are defined by the human broadly neutralizing monoclonal Abs PG9 (anti-V1/V2 quaternary epitope), PGV04 (anti-CD4bs antibody), and PGT126 (anti-glycan antibody/V3 loop). Levels of antibodies targeting the nonneutralizing anti-CD4bs antibody defined by b6 were also assessed. Week 22 serum from immunized rabbits (2 weeks postboost 2) was preincubated with solutions of biotinylated BG505 L111A or 16936-V55 (Fig. 7) gp120 Env proteins. Subsequently, the antibody-gp120 mixtures were added to wells containing multispot arrays of 4 different neutralizing monoclonal antibodies to determine if the rabbit antiserum inhibited gp120 capture by the antibodies immobilized on the plate surface. As shown in Fig. 8, we detected antibodies in the serum that competed to various degrees with each plate-bound bNAb for binding to gp120. Using the BG505 L111A antigen in the CBA, we found that the titers of antibodies competing with PG9, PGT126, and b6 epitopes elicited by the BG505 L111A DNA prime-protein boost group were significantly higher than those elicited by the control 16936 DNA prime-protein boost group ($P = 0.03$, Mann-Whitney test). Titers competing with PGV04 were ~3-fold higher for the BG505 L111A group but did not reach statistical significance. When we used a heterologous antigen (16936-V55) to measure CBA titers, we found that sera from the BG505 L111A DNA prime-protein boost group had titers to 3 regions of vulnerability that were 2- to 4-fold higher than those elicited by the 16936 DNA prime-protein boost group. Furthermore, CBA titers elicited in the BG505 L111A reverse prime-boost group were significantly higher than those detected in the 16936 protein prime-DNA boost group ($P = 0.03$, Mann-Whitney test).

DISCUSSION

HIV-1 gp120 and gp41 are targets for neutralizing antibodies. These proteins are initially synthesized as one precursor protein (gp160) that is cleaved. Heterodimers of noncovalently interacting gp41/gp120 are arranged in trimers to form antigenic spikes on the viral envelope outer surface. These trimeric spikes bind to the CD4 receptor and the CCR5 coreceptor to mediate infection of $CD4^+$ T cells. Unlike the attachment protein found on many other viruses, the HIV Env spike is incorporated on the surface of virus particles at very low levels (50). In addition, infected cells are thought to produce incomplete or incorrectly folded Env proteins, which also can be incorporated into progeny virus particles (51). The dearth of functional Env trimers is thought to be one factor that slows or prevents development of bNAbs in many HIV-

16936-16055V123loop (gp120)

MPMGSLQPLATLYLLGMLVASVLAAGNLWVTVYYGVPVWKEAKTTLFCASDAKAYETEVHNVW
ATHACVPTDPNPQELVLENVTENFNMWRNDMVDQMHEDVISLWDQSLKPCVKLTPLCV TLECR
QVNTTNATSSVNVTNGEEIKNCSFNATTEIRDKKQKVYALFYRLDIVPLEEERKGNSSKY RLINCN
TSAITQACPKVNFDPIPIHYCTPAGYAILKCNNKTFNGTGPCSNVSTVQCTHGIKPVVSTQLLLNG
SLAEEGIIIRSENLTDNVKTIIVHLEEPVEIV CTRPNNNTRKSIRIGPGQTFYATGDIIGNIRQAYCN IS
EAKWNETLQNVTKKLKEHFPNKTIIFNSSSGGDLEITTHSFNCRGEFFYCNTSKLFNGIYNGTQSN
SSNSNSTIIIPCKIKQIVNMWQKVGRAMYAPPIAGNITCTSNITGLLLVRDGGPDNVTEIFRPGGGD
MRDNWRSELYKYKVVEIKPLGIAPTGAKRRVVEGAHHHHHH

FIG 7 Protein sequence 16936-V55, which is a recombinant HIV clade C Env protein with the backbone from Env 16936 and the V1, V2, and V3 loops (red font) from Env 16055.

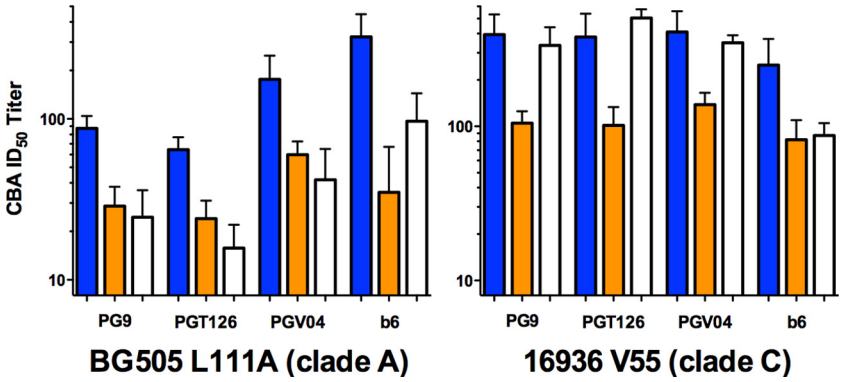

FIG 8 Analysis of vaccinated rabbit serum by competition antibody binding assay. Week-22 serum (Fig. 5) from rabbits primed with 2 doses of BG505 L111A DNA and boosted with 2 doses of BG505 L111A gp120 protein (blue bars), primed with 2 doses of 16936 gp120 DNA and boosted with 2 doses of 16936 gp120 protein (orange bars), or primed with 2 doses of BG505 L111A protein and boosted with BG505 L111A gp120 DNA (white bars) was analyzed by CBA. ID_{50} titers were determined using the MSD multiplexed ECL assay with antibodies bound to the plate and biotinylated BG505 L111A gp120 antigen (A) or 16936-V55 antigen (B) in the solution phase. Data are presented as the reciprocal of the antiserum dilution that inhibited 50% of labeled gp120 capture by monoclonal antibodies immobilized on the plate.

infected individuals (52, 53). Also, the Env protein mutates rapidly to evade host immunity, misdirecting the immune system to produce strain-specific antibodies that have limited breadth (54). As a result, most of the antibodies generated against the Env protein do not inhibit virus entry (55).

Vaccines based on HIV-1 Env subunit proteins or peptides have failed to generate antibodies that neutralize a significant spectrum of HIV variants. Eliciting antibodies that neutralize a wide range of HIV isolates is essential since there is extensive genetic diversity in circulating isolates (53, 56–58). Traditional methods of vaccine development have either been ineffective or are not feasible from a safety standpoint (59). Thus, nontraditional structure-based methods (60) involving subunit-based, peptide-based, or protein scaffold-based immunogens are being investigated by many laboratories. Experimental vaccines based on these approaches have elicited antibodies in vaccinated animals that recognize the target Env epitope, but broad virus neutralizing activity has not been observed.

With the recent availability of an expanded panel of bNAbs from several sources, it is apparent that not all HIV Env proteins are equally antigenic. In fact, very few, if any, soluble Env proteins bind to the full complement of available monoclonal bNAbs (C. R. King, unpublished data). We reasoned that Env proteins originating from bNAb donor subjects would be a reasonable starting point for vaccine development; however, it is often the case that viruses isolated from such donors have already developed resistance to their own neutralizing antibodies. In the case of the PG9 donor subject (41), we utilized bioinformatics tools to identify the MRCA sequence from 19 Env sequences derived from this individual and then searched the Los Alamos database for the best match. From this analysis, we identified a clade A Env (BG505) that binds to bNAbs representative of most of the known neutralizing antibody classes. We found that the monomeric gp120 form of this Env binds to conformation-specific antibodies (PG9 and PG16), suggesting that the soluble monomer retains certain structural features of the native trimer. Compared to BG505, the gp120 form of previously identified PG9/PG16-sensitive Envs has lower affinity for antibodies that bind preferentially to trimers, especially PG16 (61). The BG505 sequence also has asparagine in position 160, which is a requirement for binding to quaternary structure-specific PG9-like broadly neutralizing antibodies but not to isolate-specific neutralizing antibodies such as MAb 2909 (26). Substitution of leucine for alanine at position 111 (L111A) did not alter the antigenicity of the gp120 Env, nor did it change the sensitivity to neutralization with our panel of bNAbs; however, it did enable production of more stable gp120 monomers, an important consideration in vaccine development.

From the crystal structure of PG9 with variable regions 1 and 2 as well as mutation analyses, glycosylation at position N160 as well as at position N156 is required for binding to PG9 (20, 25). However, 17 of 19 Env sequences from the PG9 donor have retained potential N glycosylation (PNG) sites at position 160, and all retain N156 (Table 4), suggesting that a substitution at one of these sites is not the primary cause for neutralization resistance to PG9. Alteration of basic amino acids within strand C of V1/V2 has been shown to affect sensitivity to neutralization by PG9 and related antibodies (62). Consistent with these observations, analysis of the 19 available sequences from subject 024 revealed that they all differed from BG505 at positions 169 and 171 in strand C, which are known to be involved in forming hydrogen bonds with PG9 (25). Thus, neutralization resistance of subject 024-derived viruses to PG9 may result primarily from mutations in the β-sheet (strand C) rather than those in PNG sites.

Glycosylation at position N332 is thought to be required for binding and neutralization by 2G12 and several antibodies from the new PGT series (21). Interestingly, BG505 binds to most glycan-dependent antibodies (including PGT126) even though it lacks a PNG site at position 332. Neutralization of the BG505 pseudovirus by PGT126, which is closely related to PGT128, is surprising since structural studies of cocrystals formed with PGT128 FAb and an outer domain construct containing a mini-V3 loop (eODmV3) indicate that there is binding to a glycan determinant at position N332 (24) which is absent in BG505. Binding and neutralization of BG505 by PGT126 may be explained by promiscuity of some antibodies (25) or regulation of access to the V3 loop by V1V2 (63). In the current study, we found

TABLE 4 Alignment of Env sequences from subject 024 with the BG505 sequence spanning the PG9 epitope region

Sequence ID	Alignment (based on HXB2 convention)[a]
	126 130 156 160 170 180 185 186 190 196
BG505	CVTLQCT------NVTNNITDDMRG---ELKNCSFNMTTELRDK**K**Q**K**VYSLFYRLDVVQINENQGNRSNNS---NKEYRLINC
MRCA	CVTLNCS----DHKISINSTTNNLTETGEMKNCSYNMTTELRDKTQQVYSLFYRLDIVPVNEKQSNSSSNNSSNNSEYRLINC
064V1_011	CVTLNCS-----HKILINTTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--DSEYRLINC
064V1_020	CVTLNCS-----HRILINDTEKNVT---DMRNCSYRITTELRDKTQQVYSLFYRLDIVPVKEKQSNSSNKSSS-DNEYRLINC
064V1_021	CVTLNCS-----HKILINTTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--DSEYRLINC
064V1_026	CVTLNCS-----HKILINFTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--NSEYRLINC
064V1_032	CVTLNCS-----HKILISSTEKSVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--NSEYRLINC
064V1_034	CVTLNCS-----HKILISSTEKSVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--NSEYRLINC
064V1_035	CVTLNCS-----HKILINTTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--DSEYRLINC
064V1_038	CVTLNCS-----HKILINFTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGRNSS--NSEYRLINC
064V1_039	CVTLNCS-----HKILIKSTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSGSNSS--NSEYRLINC
064V1_046	CVTLNCS-----HKILINTTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSNSRSNSN--NSEYRLINC
064V2_003	CVTLNCS-----HRILINDTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSKSSSNSN--NSEYRLINC
064V2_006	CVTLNCS-----HKILINNTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVKEKQSSSSNSS---YSDYRLINC
064V2_010	CVTLNCS-----HKILINNTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVKEKQSSSSNSS---YSDYRLINC
064V2_016	CVTLNCS-----HKILINNTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVKEKQSSSSNSS---YSDYRLINC
064V2_017	CVTLNCS-----HRILINDTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVNEKQSKSSSNS---NSEYRLINC
064V2_021	CVTLNCSYEVNATYSNLNLTENMRE---EIKNCSYNMTTELRDKTQQVYSLFYRLDIVPVKEKQSDSSNDSSS-KNIYRLINC
064V2_033	CVTLNCS-----HRILINDTEKNVT---DMRNCSYRITTELRDRTQQVYSLFYRLDIVPVKEKQSNSSNNSSS-KNEYRLINC
064V2_036	CVTLNCS-----HKILINNTEKNVT---DMRNCSYNITTELRDKTQEVYSLFYRLDIVPVKEKQSSSSNSS---YSDYRLINC
064V2_047	CVTLVCNDNIISINASTNATQNNVTDTGEMKNCSYNMTTELRDKTQQVSSLFYRLDIVPVNEKQSNSSNNSSS-N

[a] The amino acids at positions 169 and 171 are shown in bold in the BG505 sequence to highlight differences with all other sequences at these positions.

that substitution of BG505 threonine 332 with asparagine restored binding of the PGT135/PGT136 antibody family to the level of other PGTs. Importantly, this substitution restores neutralization sensitivity to PGT135 (64) and produced a gp120 Env that bound efficiently to all classes of bNAbs tested, except for PGT145, which is highly selective for a determinant found in the trimeric conformation.

Of note, 2 neutralizing antibodies that bound to BG505 L111A monomers (b12, 2G12) failed to neutralize the BG505 pseudovirus (see Table 1). Thus, there is not a strict correlation between antigenicity of Env proteins and neutralization of pseudoviruses expressing the corresponding Env (BG505). Moreover, the BG505 L111A mutant also is recognized by nonneutralizing Abs like b6 and F105; thus, the BG505 L111A monomer, like most experimental Env subunit vaccines, has the potential to elicit antibodies to immunodominant nonneutralizing epitopes. Nevertheless, it is encouraging that BG505 L111A gp120 administered in a prime-boost regimen elicited antibodies that neutralized multiple heterologous tier 1 and tier 2 viruses and that were able to compete with bnAbs specific for 3 distinct regions of vulnerability on the HIV Env, the PG9 epitope, the PGT126 epitope, and the CD4bs. Thus, the presence of nonneutralizing epitopes on the BG505 L111A immunogen did not preclude elicitation of antibodies against other epitopes on Env that are targets for neutralizing antibodies. However, the CBA results must be interpreted cautiously, because it may be detecting antibodies binding to epitopes that are in close proximity but distinct from the sites of vulnerability recognized by the bNAbs. Nevertheless, when the CBA was conducted with a clade-mismatched gp120 Env (16936-V55), antibodies specific for conserved determinants on the Env protein were detected. Importantly, the BG505 L111A immunogen elicited higher neutralizing antibody titers to both tier 1 and tier 2 viruses than the control (16936) immunogen, suggesting that the presence of the PG9 epitope may have contributed to elicitation of a neutralizing antibody response. The CBA data support the concept that the BG505 L111A gp120 immunogen can elicit a robust antibody response to the PG9 epitope as well as to other conformation-dependent antigenic sites on Env, suggesting that this Env sequence is a reasonable starting point for development of subunit or viral vector-based vaccine candidates and for structural studies of protein trimers that preserve essential features of the native Env spike (64).

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